AN INHIBITOR OF ANGIOGENESIS ISOLATED FROM BOVINE CORNEA

ELIZABETH ANNE MULLEN

1992

AN INHIBITOR OF ANGIOGENESIS ISOLATED FROM BOVINE CORNEA

Elizabeth Anne Mullen

A thesis in partial fulfillment of the requirements for the degree

Doctor of Medicine

Yale University School of Medicine
Department of Orthopaedics and Rehabilitation

1992

DEDICATION

With gratitude and love to my parents, Katherine and John Mullen, for their boundless support and encouragement.

TABLE OF CONTENTS

ABSTRACT

AN INHIBITOR OF ANGIOGENESIS ISOLATED FROM BOVINE CORNEA. Elizabeth A. Mullen, Rosalind A. Rosenthal, *Peter V. Hauschka and Judah Folkman. Department of Surgical Research, Children's Hospital, Boston, MA, *Department of Orthopedic Research, Children's Hospital, Boston, MA. (Sponsored by Caren M. Gundberg, Department of Orthopaedics and Rehabilitation, Yale University School of Medicine, New Haven, CT).

The normally avascular cornea paradoxically contains stores of a potent stimulator of angiogenesis, basic fibroblast growth factor (bFGF) (Am. J. Path. 130:393, 1988). Mun *et al.* reported the partial purification from bovine cornea of a low molecular weight molecule which inhibits angiogenesis in the chick embryo (Inv Opth & Vis Sci. 30:151, 1989). This factor, termed corneal angiogenesis inhibitor (CAI), was also shown to antagonize certain bFGF induced effects on capillary endothelial cells, including cell migration, cell spreading and cell rounding. Endothelial cell rounding *in vitro* has been correlated with anti-angiogenesis *in vivo* (Endocrinology 119:1768, 1986). We report an improved purification method for CAI, which like Mun *et al.* used the ability of CAI to cause endothelial cell rounding to direct purification. We also measured the ability of CAI to inhibit DNA synthesis in endothelial cells, and block angiogenesis on the chorioallantoic membrane of chick embryos. Our improved

purification produced a sample sufficiently pure for preliminary structural analysis. This method includes: (1) Dissection of bovine corneas; (2) Extraction into 100°C distilled water; (3) Filtration of corneas and centrifugation of filtrate; (4) Ultrafiltration through pressure dialysis, 1000 molecular weight (MW) cutoff, and lyophilization; (5) Extraction in 80% dimethylformamide (DMF), centrifugation and evaporation of solvent; (6) 100% DMF extraction, centrifugation and evaporation of solvent; (7) Reversed phase high performance liquid chromatography on both a C18 semi-preparative column and a C18 analytical column. Samples purified by this method were analyzed by desorptive chemical ionization and electospray mass spectroscopy. Possible candidates for the CAI molecule were identified at MW 228 and 172. CAI may play an important role in the regulation of vascularization in the cornea, perhaps acting as an endogenous inhibitor of bFGF.

INTRODUCTION

The regulation of vascularization is of great importance in many normal physiologic and pathologic processes. The term "angiogenesis" was introduced in 1935 to describe the formation of new blood vessels in the placenta (Hertig, 1935). It is currently used to denote the process of generating new capillary blood vessels from established vessels, resulting in "neovascularization". Under normal physiologic conditions, angiogenesis has an essential role in embryonic development, ovulation, placental maturation, and wound healing (Jakob *et al.*, 1977; Gospodarowicz and Thakral, 1978; Wagner, 1980; Bär, 1980; Hunt *et al.*, 1981).

Abnormal neovascularization dominates a number of serious pathologic conditions, both malignant and nonneoplastic. Much evidence exists that solid tumor growth is angiogenesis dependent (reviewed in Folkman, 1990; Klagsbrun and Folkman, 1990). Tumor angiogenesis and the incidence of metastasis in invasive breast carcinoma are highly correlated (Weidner *et al.*, 1991). Many diverse diseases (Table 1) in which the dominant pathology is persistent neovascularization have been categorized as "angiogenic disease" (Folkman and Klagsbrun, 1987). Patients affected by angiogenic diseases span a wide range of ages, and include both sexes. Care and management of these conditions are divided among numerous medical and surgical specialties. For example, the excessive proliferation and shedding of the

epidermis in psoriasis has been related to abnormal capillary growth in the dermis (Ryan, 1970). Hemangiomas can occur in newborn babies, with abnormal capillary proliferation persisting until up to two years of age, and which in rare cases may result in death from hemorrhage (Folkman, 1984; Folkman and Klagsbrun, 1987). All hemangiomas are known to eventually regress spontaneously; the mechanism through which this occurs is completely unknown. Atherosclerotic plaques can bleed secondary to capillary proliferation within the vessel wall, and contribute to sudden occlusion of coronary arteries (Barger *et al.*, 1984).

Abnormal neovascularization can also affect normally avascular tissue, as in rheumatoid arthritis, where the occurrence of capillary growth destroys joint cartilage (Matsubara and Ziff, 1987; Koch *et al.*, 1986; Crisp *et al.*, 1984). Neovascularization in avascular components of the eye leads to decreased vision or blindness. Diabetic retinopathy, retrolental fibroplasia, and neovascular glaucoma are pathologic conditions which involve a breach of the vascular/avascular barriers within the eye (Klagsbrun and Folkman, 1990). Disorders involving either the corneal epithelium or stroma (Table 2) can result in invasion of blood vessels into the avascular corneal stroma, and are a leading cause of blindness worldwide (Garner, 1986).

The control of the stimulation and inhibition of angiogenesis has been extensively investigated. A picture has

emerged of a regulatory system analogous to other known physiologic processes, such as blood coagulation (Folkman and Klagsbrun, 1987). Both systems must be maintained in a state of inactivity, but constant readiness, over a long period of time. The proliferation of vascular endothelial cells in most normal adult tissue is measured with turnover time in years (Denekamp, 1984), congruent with a microvascular system designed to remain quiescent over periods of weeks to decades. The microvascular system, however, must be capable of prompt response when faced with pathologic conditions or physiologic demands which require the rapid growth of new vessels.

The search for factors which may stimulate or inhibit angiogenesis has been extensive. A number of factors, including polypeptides and nonpeptide low molecular weight compounds, have been shown to be angiogenic *in vivo* (Klagsbrun and Folkman, 1990) (Table 3). The process of angiogenesis *in vivo* is very complex. Many sequential steps are required: the degradation of capillary basement membrane, the migration and proliferation of endothelial cells, and tube formation. One of the earliest described stimulators of cell division, basic fibroblast growth factor (bFGF) has been found to be a potent stimulator of endothelial cell growth and angiogenesis. The term FGF was first used to describe a cationic polypeptide isolated from brain in 1974 (Gospodarowicz, 1974, 1975); its primary amino acid structure was described in 1985 (Esch *et al.*, 1985). An anionic species of FGF, which was termed acidic fibroblast growth factor

(aFGF) was also found in brain, and shown to have a 53% absolute sequence homology to the cationic polypeptide, which became known as bFGF (Thomas *et al.*, 1980; Lemmon and Bradshaw, 1983; Thomas 1987; Esch *et al.*, 1985). The FGFs have since been shown to be mitogenic and chemotactic for endothelial cells *in vitro*, to stimulate endothelial cells to produce proteases (collagenase and plasminogen activator) which result in degradation of basement membrane, and to induce capillary endothelial cells to migrate into three-dimensional collagen matrices and form tubes similar to capillaries (Gross *et al.*, 1983; Montesano *et al.*, 1986). These demonstrated *in vitro* properties of the FGFs are consistent with their observed angiogenic activity *in vivo*.

The FGFs are among the most ubiquitous growth factors in the body (Folkman and Klagsbrun, 1987). bFGF is found in brain (Lobb and Fett, 1984; Gospodarowicz *et al.*, 1984; Bohlen *et al.*, 1984), eye (Courty *et al.*, 1985), cartilage (Sullivan and Klagsbrun, 1985), bone (Hauschka *et al.*, 1986), reproductive tissue (Moscatelli *et al.*, 1986), adrenal gland (Gospodarowicz *et al.*, 1986), kidney (Baird *et al.*, 1985a), macrophages (Baird *et al.*, 1985b) and tumors (Shing *et al.*, 1984; Klagsbrun *et al.*, 1986), while aFGF is present mainly in neural tissue including brain (Lobb and Fett, 1984; Thomas *et al.*, 1984; Conn and Hatcher, 1984), retina (D'Amore and Klagsbrun, 1984), and eye (Courty *et al.*, 1985), and has also been identified in bone (Klagsbrun and Folkman, 1990). This extensive distribution raises the question of how rampant capillary proliferation is prevented in both

normally vascular, and particularly, in normally avascular tissue. This question, in turn, has prompted the search for naturally occurring inhibitors of angiogenesis.

The first angiogenesis inhibitor was found in the normally avascular cartilage (Eisenstein *et al.*, 1973; Brem and Folkman, 1975), and has recently been characterized and identified as cartilage-derived inhibitor (CDI) (Moses *et al.*, 1990). CDI is a member of the tissue inhibitor of metalloproteinase (TIMP) family, and has been shown to inhibit angiogenesis *in vivo*, and capillary endothelial cell proliferation and migration *in vitro* (Moses and Langer, 1991; Moses *et al.*, 1990). This protein is also an inhibitor of mammalian collagenase. A number of other naturally-occurring inhibitors of angiogenesis have been identified. These factors include the 16 kDa fragment of prolactin (Ferrara *et al.*, 1991), thrombospondin (Good *et al.*, 1990; Bagavandoss and Wilks, 1990; Taraboletti *et al.*, 1990), platelet factor 4 (Maione *et al.*, 1990), and protamine (Taylor and Folkman, 1982). Transforming growth factor-beta (TGF-B) (Baird and Durkin, 1986; Müller *et al.*, 1987; Jennings *et al.*, 1988), and tumor necrosis factor-alpha (TNF-α) (Frater-Schroder *et al.*, 1987; Schweigerer *et al.*, 1987) are both polypeptides that inhibit endothelial cell proliferation *in vitro*, yet promote angiogenesis *in vivo* and tube formation *in vitro* . It is possible that TNF-α and TGF-B may inhibit the proliferative phase of endothelial cells, but promote the differentiation phase, e.g., matrix production and tube formation (Klagsbrun and Folkman, 1990).

To date, none of these known angiogenesis inhibitors have been found in the avascular structures of the eye. However, much evidence exists to support the occurrence of endogenous inhibition of angiogenesis within the eye.

The eye contains both vascular and avascular structures (Figure 1). The sclera, which comprises the posterior four-fifths of the surface of the globe, is composed largely of collagen, proteoglycan and fibroblasts. Interestingly, although the sclera is interposed between two highly vascular tissues, the episclera and the choroid, it is avascular. Inflammatory diseases of the ocular surface involving macrophages and bFGF containing mast cells, which often result in corneal neovascularization, do not cause scleral neovascularization (Sunderkotter *et al.*, 1991). This could be attributed to the presence of a potent endogenous inhibitor(s) in the sclera. Alternatively, there could be physical or mechanical resistance to angiogenesis in the sclera.

The fetal lens contains hyaloid vessels during embryonic development; these regress shortly before birth. Human lens epithelium has been shown to contain angiogenic aFGF (Baudouin *et al.*, 1990). Paradoxically, extracts of both human and bovine lens demonstrate a reversible dose-dependent inhibition of bovine aorta endothelial cell proliferation *in vitro*. This result suggests that an extractable, endogenous inhibitor of angiogenesis also exists (Williams *et al.*, 1984). It has also been shown that the removal of lens and capsule in proliferative diabetic retinopathy

leads to an increased incidence of iris neovascularization (Aiello *et al.*, 1983). Numerous mechanisms to explain this phenomenon have been postulated, including facilitated diffusion of a retinal "vasoformative factor" (Ashton, 1957), decreased oxygenation of the iris and release of an "iris vasoformative factor" (Schulze, 1967), and reduced endogenous inhibition from the lens (Williams *et al.*, 1984).

The aqueous humor fills the anterior chamber, and physically contacts the cornea, lens, iris, ciliary body and trabecular meshwork. It has been demonstrated that normal aqueous humor inhibits capillary endothelial cell proliferation *in vitro*, while it promotes a dose-dependent proliferation of 3T3 smooth muscles cells and dermal fibroblast *in vitro* (Okamoto *et al.*, 1990). Interestingly, it was also found that this anti-angiogenic activity is absent in the aqueous humor of diabetic rabbits. The identity or source of the proposed endogenous inhibitor has not been elucidated.

The vitreous humor occupies the chamber anterior to the retina and posterior to the lens. It is composed of water, collagen, hyalocytes, and the proteoglycan hyaluronic acid. Clinically, the vitreous appears to be resistant to angiogenesis. In proliferative diabetic retinopathy, fronds of developing blood vessels grow onto the vitreous surface, but do not penetrate its substance. Interestingly, the vitreous from patients with diabetic proliferative retinopathy has been shown to contain

elevated levels of bFGF (Sivalingam *et al.*, 1990). This observation suggests the possibility of an increase in the ratio of angiogenesis stimulators to inhibitors in the vitreous in this disease. As evidence of the angiogenic potential of the vitreous, degradation products of vitreous hyaluronic acid assayed on the chick chorioallantoic membrane (CAM) were shown to induce neovascularization (West *et al.*, 1985). However, vitreous has also been shown to be inhibitory of angiogenesis. Angiogenic tumors implanted into the vitreous remain small and avascular - unless they happen into direct contact with the retina (Brem *et al.*, 1976). Extracts from the vitreous have been shown to inhibit both retinal extract-induced angiogenesis, and tumor-induced angiogenesis (Lutty *et al.*, 1985). These findings support the concept of regulatory interplay between both inhibitory and stimulatory factors of angiogenesis which, under normal conditions, maintain an avascular state in the vitreous.

Neovascularization of the retina is a serious consequence of several pathologic conditions; diabetes, sickle cell anemia, branch vein occlusion, and retrolental fibroplasia (Patz, 1982). Blindness results from vitreous hemorrhage and retinal detachment following the development of new blood vessels in the retina (Ashton, 1983). Studies have shown that retina contains endothelial cell growth factors, including aFGF and bFGF (Baird *et al.*, 1985), as well as serum factors such as platelet release factors (Miyazono *et al.*, 1987), and insulin-like growth factors (Grant *et al.*, 1987; King *et al.*, 1985). However, more

recently McIntosh and co-workers showed that retinal extract inhibited the serum-induced proliferation of retinal capillary endothelial cells (McIntosh *et al.*, 1989). This finding suggests a regulatory system for vascularization within the retina involving a balance of angiogenesis stimulators and inhibitors.

The normally avascular cornea is paradoxically known to contain large stores of angiogenic bFGF (Folkman *et al.*, 1988). The bFGF has been shown to bind to Bowman's layer, the acellular, most anterior portion of the collagenous corneal stroma, which is rich in proteoglycan (Adamis *et al.*, 1991). BFGF is also found in Descemet's membrane, a true basement membrane produced by corneal endothelial cells on the posterior surface of the cornea (Figure 2). Folkman and co-workers demonstrated that bFGF could be released from the cornea by treatment with heparin, heparan sulfate, or heparanase (Folkman *et al.*, 1988). Their work suggested that sequestration of this angiogenic endothelial mitogen in the basement membrane could be a general mechanism for controlling the accessibility of bFGF to vascular endothelium, therefore regulating vascularization. The abnormal release of bFGF could be the cause of corneal vascularization in a number of ocular diseases. In support of this concept, injury-induced release of bFGF from corneal epithelial cells has recently been demonstrated (Adamis *et al.*, 1991).

As an alternate, or additional explanation for the maintenance of avascularity of the cornea in the presence of

angiogenic bFGF, the existence of an angiogenesis inhibitor in the cornea was hypothesized. A number of studies supported this hypothesis. Corneal extracts which were mitogenic for endothelial cells were found to be inhibitory of cell growth at high concentrations (Folkman *et al.*, 1988). When pieces of cornea from several animal species were placed onto the chorioallantoic membrane (CAM) of a developing chick embryo (Folkman, Casey, Levenson, unpubl. results), avascular zones appeared around the corneas. These corneas could be moved to another chick embryo, and again, an avascular zone on the CAM surrounding the cornea would be produced. This suggested the production of a diffusible angiogenesis inhibitor by the cornea.

A systematic search for this corneal angiogenesis inhibitor, termed CAI, was undertaken. A partial purification scheme was established by Susan Doctrow, Edward Mun, and Judah Folkman, and many characteristics of the molecule defined. The inhibitor was found to stable to boiling and pronase digestion. Sensitive amino acid analysis techniques revealed no amino acids, indicating the inhibitor is not a peptide. Bioactivities of the partially purified CAI were also characterized. In addition to the inhibition of angiogenesis on the CAM assay, CAI was shown to cause inhibition of DNA synthesis in endothelial cells, and inhibition of bFGF stimulated cell migration, in a Boyden chamber model (Mun *et al.*, 1989). Partially purified CAI blocks both attachment (cell number analysis) and cell spreading of bovine capillary endothelial (BCE) cells in response to bFGF

(morphometric analysis) (Carter, Ingber, Folkman, unpubl.). Staining with antibodies to vimentin demonstrates disconnection of the intermediate filaments from the plasma membrane, followed by the depolarization of actin. Associated with the inhibition of cell spreading, corneal extracts can also cause endothelial cells to "round", in a dose-dependent manner. This rounding effect can be reversed by titration with bFGF. Rounded cells exclude trypan blue, and stain with fluorescein diacetate, indicating viability. Inhibition of cell migration was observed with the lowest quantities of CAI, followed by DNA synthesis inhibition, and endothelial cell rounding. Angiogenesis inhibition on the CAM required the most material.

A semi-quantitative screening assay based on the cell rounding effect of CAI was designed to guide the further purification of this molecule (Mun *et al.*, 1989). In large part, the purification was hampered by inconsistent behaviors and reproducibility of results, particularly in achieving binding to high-pressure liquid chromatography (HPLC) column resins.

In our work, we have further improved the purification of CAI, while retaining its bioactivity. Aqueous extraction, ultrafiltration, organic extraction, and reverse phase high-pressure liquid chromatography were used to reproducibly obtain a partially pure preparation of CAI, suitable for preliminary analysis by mass spectroscopy.

METHODS

Cell Culture - Bovine capillary endothelial (BCE) cells were isolated from bovine adrenal glands as described by Folkman *et al.*, (1979), except 5 μ/ml crude retinal factor (Gitlin and D'Amore, 1983) was used instead of mouse sarcoma conditioned medium. BCE cells are usually maintained in Dulbecco's modified Eagle's medium (DMEM), containing 25 μg/ml endothelial mitogen (BTI), 10% calf serum, 2 mM glutamine, and antibiotics.

Endothelial Cell Rounding Assay - The samples to be tested on the rounding assay were first dried in a model TM 100 SpeedVac Concentrator (Savant), and then dissolved in rounding assay media (DMEM, 10% calf serum, 20 mM HEPES pH 7.4, 2 mM glutamine and antibiotics). Bovine capillary endothelial cells were seeded onto 96 well half-area plates (0.16 cm^2 wells) (CoStar), at a density of 2000 cells per 25 μl per well, in rounding assay medium. After a 6 - 12 hour attachment period, the cells were treated with serial dilutions of inhibitor fractions (Figure 3). Cells were examined by phase microscopy after 6 - 24 hours, and judged for cell rounding. One unit of cell rounding is defined as the amount of sample which is sufficient to cause rounding in 50% of the cells in a given well (Figure 4). The number of rounding units in a given sample is calculated from the greatest dilution of sample which will produce one unit of cell rounding.

DNA Synthesis Assay - Bovine capillary endothelial cells are plated on 48 well plates (CoStar) at a density of 7500 cells/well in DMEM containing 2% calf serum, 3 μM thymidine, 2 mM glutamine, and antibiotics. After a 24 hour attachment period, the medium is exchanged with rounding assay medium, the samples are added, and the cells are incubated for an additional 18 hours at 37°C. The cells are then incubated with 1 μCi ^{3}H-thymidine/well (82.2 Ci/mmol) for 6 h. The cells are washed once with phosphate-buffered saline (PBS). The cells are subjected twice to a wash of cold methanol for 5 minutes each wash, and twice to cold 5% trichloroacetic acid for 10 minutes each. The plates are washed briefly with water and O.3 M NaOH is added. The NaOH-solubilized material is transferred to vials containing scintillation fluid. The sample is neutralized with glacial acetic acid, and the vials are counted in a Beckman model LS 3801 scintillation counter.

Chick Chorioallantoic Membrane (CAM) Assay - The CAM assay is performed as described by Crum *et al.* (1985). The sample to be tested for stimulatory or inhibitory properties is combined with a carrier of 0.45% methylcellulose (10 μl), and air dried to form a disc of 2 mm diameter. This disc is applied to the shell-less embryo in a cultured petri dish (Auerbach *et al.*, 1974; Klagsbrun *et al.*, 1976; Folkman, 1985). After incubation at 37°C in 3% $C0_2$ for 48 hours, the embryos are examined under a dissecting microscope (6X power). An embryo is scored as negative (indicating inhibition) if an avascular zone larger than 4

mm^2 is observed (Figure 5). Positive angiogenic activity is revealed by the radical ingrowth of new vessels. Inhibition or stimulation of neovascularization can be confirmed by histologic microsections of India-ink-injected specimens (Haudenschild, 1980).

PURIFICATION SCHEME

Dissection - Bovine eyes (-70°C), with the eyelids removed, were obtained from BioResources, Texas, and the corneas were dissected immediately upon arrival. The eyeballs were rinsed briefly in cold distilled H_2O, and the corneas carefully excised from the frozen eyes, using a number 15 scalpel blade (Figure 6). The corneas were placed on dry ice, and stored at -80°C until use.

Extractions - To begin the extraction of CAI, frozen corneas (500) were placed in a 1 liter Erlenmeyer flask, with 500 ml of HPLC grade H_2O (Pierce HPLC grade solvents were used exclusively throughout the purification). The flask was put into a hot water bath, brought to 100°C, and boiled for 20 minutes. The hot extract was filtered through coarse Whatman filter paper under vacuum, to remove the corneas and debris. The filtrate was centrifuged for 15 minutes at 10,000xg, and the supernatant retained. The supernatant was subjected to ultrafiltration using an Amicon YM2 (1000 molecular weight cut-off) membrane under 55 psi nitrogen pressure, overnight at 4°C. The filtrate was

collected and lyophilized. The lyophilized powder was reconstituted with 10 ml of H_20. Dimethylformamide (DMF, 90 ml) was added, and the solution stirred at room temperature for 1 hour. The material was then centrifuged in glass tubes for 20 minutes at IO,OOOxg. The supernatant was dried in a model EL 131 RotoVapor (Buchi). DMF (25 ml) was added directly to the round bottom RotoVapor flask, and the flask agitated at room temperature for 1 hour. This material was centrifuged for 20 minutes, and the supernatant retained and stored at 4°C.

High-Pressure Liquid Chromatography

C18 Analytical HPLC column - Initial investigation of the binding and elution of CAI was performed using a Supelco octadecylsilica (C18 reversed phase) analytical (15 cm X 4.6 mm) HPLC column. DMF extracted material (1 ml) was dried in 12 X 75mm borosilicate tubes in a SpeedVac rotor (Savant), and resuspended in 100 µl of H20, 0.1% trifluoroacetic acid. This sample was injected onto the column, and washed with a complex gradient of O -100% acetonitrile, 0. 1% trifluoroacetic acid (TFA) (Figure 7). Fractions (2 ml) were collected, and dried in a Speed Vac. Absorbance of the fractions eluting from the column was monitored at 220nm. These fractions were saved for assay in the endothelial cell rounding assay, DNA synthesis inhibition, and CAM assay.

C18 Semi-preparative HPLC column - A similar technique was employed when using the C18 semi-preparative HPLC column.

The 100% DMF extracted material (3ml) was dried and resuspended as above, in 0.1% TFA (200 µl). This sample was injected onto a C18 semipreparative (25 cm X IOmm) HPLC column (Supelco), and absorbance of eluted fractions again measured at 220 nm (Figure 8). Fractions were collected and assayed for bioactivity.

C18 Analytical HPLC column - second HPLC purification step. A single fraction (fraction 13) obtained from the C18 semi-preparative column which demonstrated the greatest number of units of endothelial cell rounding activity was selected and dried. This fraction was reconstituted in 100 µl of H20, injected onto a Supelco C18 analytical (15 cm X 4.6mm) HPLC column (Supelco), and eluted with a complex gradient of O - 50% acetonitrile, in the absence of trifluoroacetic acid (Figure 9). Fractions were collected by hand, as individual peaks of absorbance seen at 220 nm. These samples representing individual peaks of absorbance were assayed for bioactivity using the endothelial cell rounding assay, and the elution fractions which contained bioactivity were determined. This second HPLC purification step was repeated under exact conditions, using identical starting sample material. Identical (superimposable) chromatographs were obtained. These fractions were similarly collected by hand, as individual peaks, and retained for mass spectroscopy investigations (Figure 10).

Mass Spectroscopy - As detailed in the previous section, fractions which eluted from the C18 analytical column

purification step identically to those which had demonstrated bioactivity were collected in pre-weighed 12 x 75 baked glass tubes, dried in a SpeedVac, and submitted for structural analysis to SmithKline Beecham Pharmaceuticals (Michael Huddleston, Steven A. Carr, Ph.D.). Additionally, the fractions representing absorptive peaks which did not demonstrate bioactivity were submitted for comparative studies. All samples were analyzed by electrospray mass spectroscopy (ESMS), and bioactive fractions were analyzed by desorptive chemical ionization (DCI) in both positive and negative ion modes, using both ammonia and isobutane as the reagent gases (Steven Carr, personal communication).

Acknowledgements - Isolation of bovine capillary endothelial cells from adrenal glands was carried out by Catherine Butterfield. Dissection of the corneas was made possible by the assistance of many laboratory members, including Nicholas Saenz, Rosalind Rosenthal, Depak Tanigrahy, Joseph Megyesi and Maria Rupnick. The DNA synthesis assay was done with the assistance of Rosalind Rosenthal. The chick embryos for the CAM assay were prepared by Jerry Jackson. The CAM assay performed, and scored by Judah Folkman. Mass spectroscopy was done at SmithKline Beecham Pharmaceuticals by Michael Huddleston and Steven Carr.

RESULTS

Previously, a method of extraction and partial purification of CAI from bovine cornea was established by Susan Doctrow and Ed Mun. A schematic of their method is given in Table 4, and in Table 5, a schematic of the improved purification method which we have established is presented. In the previous scheme, corneas purchased from BioResources were extracted in 10% ethanol, overnight in the cold. Ethanol was employed to control bacterial overgrowth. A second two hour extraction followed, and the extract was boiled for 15 minutes after which the corneas were removed by filtration. The extract was passed through a membrane with a MW cut-off of 1000, and the filtrate lyophilized. Two chloroform : methanol (50:50) extractions were performed, and the soluble fraction was retained and dried on a RotoVapor. Several conditions for high pressure liquid chromatography were employed, but successful binding and elution of the CAI activity could not be reproducibly achieved with the chloroform:methanol extracted material.

We have developed an improved purification method for CAI. In order to better control for the preparation and storage of our starting material, we obtained frozen whole bovine eyes, and dissected the corneas in our laboratory directly upon arrival from BioResources. Next, the corneas were boiled in HPLC H_20 for 20 minutes. The resultant extract yielded the same or higher amount of rounding activity/cornea as the longer cold extraction of the

previous method, but had a lower protein content, and less visible debris. Significant advantages of this step are also the reduction in time from 28 to 2 hours, and elimination of the need for ethanol, which must be removed later in the purification.

The ultrafiltration step remains unchanged in the current method. This step yields an 80% recovery of rounding activity after filtration (Table 6). We have recently found that a second ultrafiltration step, using a membrane with a 500 MW cut-off and identical conditions, yields an 80% recovery of activity that had been recovered after the first ultrafiltration step (1000 MW cut-off filter).

The major modification in the current method is the organic extraction performed. In the current method, DMF is added to a concentrated aqueous solution of the CAI, to produce a final concentration of 90% DMF. This instantly produces a large, flocculent precipitate. After centrifugation, almost 90% of the rounding activity is recovered in the supernatant, and none is detected in the pellet. A subsequent 100% DMF extraction yields more precipitated material, and 85 - 90% recovery of rounding activity. The results of these extractions compare favorably with the result of the organic extraction of the previous purification methods. The chloroform:methanol extraction yielded only a 50% recovery of activity in the soluble fraction, with a nearly equal amount of activity detectable in the insoluble material, even after two overnight extractions.

The material obtained from the DMF extractions also demonstrated the ability to inhibit DNA synthesis, as partially purified CAI by the previous method did (Figure 11). There is significant inhibition of DNA synthesis, at concentrations of CAI much less than required for rounding activity. Similar results had been found with the chloroform: methanol extracted material.

The most important outcome of the DMF extractions was that with this material, we were able to establish conditions to consistently bind and elute CAI activity from HPLC C18 reverse phase columns. Figure 7 shows a chromatograph obtained by using a C18 analytical column, and running a complex gradient from O to 100% acetonitrile, with 0.1% trifluoroacetic acid. Fractions were collected and assayed for rounding activity. Fractions 2 - 14 all showed some rounding activity, but two major peaks of activity were demonstrated. The early fraction were very acidic, with a pH of 5 in the most concentrated samples in the cell rounding assay. In order to test if all the rounding activity seen in these fractions correlated with anti-angiogenic activity, we tested the fractions on the CAM assay. The results are presented in Table 7. Fractions from the C18 column were pooled into four groups. Pool 1-4 and 10-13 showed similar amounts of endothelial cell rounding activity, the other pools less. Pool 1-4 was clearly toxic to the embryos, killing 4/5. The one surviving egg had no avascular zone. In contrast, the pool of fractions 10-13 showed dramatic anti-angiogenic activity, with 5/5 large avascular

zones. The other pools showed no toxicity, and both demonstrated some weak anti-angiogenic activity.

In order to increase the amount of CAI purified, we scaled up the purification using a C18 semipreparative column. The larger column yielded better resolution and separation, again, with two major peaks of rounding activity seen (Figure 8). Interestingly, the fractions collected from this column which were highly acidic were fraction 3 and 4, and no rounding activity was seen in these fractions.

As the next step in the purification, a single fraction from the semipreparative column containing high rounding activity was selected, and run on the C18 analytical column. A more gradual gradient of acetonitrile, without TFA, was utilized, in an attempt to obtain further separation of the bioactive material. The resultant chromatograph contained only a few well-delineated peaks, and was found to be highly reproducible, run under identical conditions, using identical starting material (Figure 9). The peaks seen on the C18 analytical chromatograph were collected by hand, and dried in a SpeedVac. Presumably identical fractions were obtained from consecutive runs: one set was devoted towards assay for endothelial cell rounding activity, and was set was prepared and retained for analysis by mass spectroscopy. The first and fifth peak (Figure 10) both showed rounding activity.

Because of the concern of possible toxicity in the bioactivity assays from the use of organic solvents throughout the purification, a blank run of the entire purification scheme was performed, and samples from each stage of the purification were tested on the endothelial cell rounding assay. All samples demonstrated no cell rounding, and no toxicity to the cells was observed. These blank samples, produced by following the purification scheme, were also submitted with the active samples for mass spectroscopy analysis, to rule out artifactual results.

The mass spectroscopy analysis showed a consistent species of molecular weight 228 in electrospray mass spectroscopy (ESMS) and desorptive chemical ionization (DCI). A gas phase exchange experiment indicated that the molecule has three exchangeable hydrogens (H). The electrospray data was clouded by very strong signals derived from sodium trifluoroacetate. A unique peak was seen at m/z 173 (possible Mr = 172). This signal, however, was not observed in the DCI, and could not be pursued further.

Electrospray and DCI mass spectra was also performed on blanks which had been submitted. These blanks were obtained by performing the entire purification scheme, without the corneas. It was concluded that the extraction process and solvents were not contributing to the observations which had been made about the active samples.

Scanning high resolution was next attempted on these partially analyzed samples, however, the sample was found to be inadequate, secondary either to insufficient quantity, or degradation of the material.

DISCUSSION

This paper describes the further characterization and purification of a novel corneal angiogenesis inhibitor, initially described by Mun *et al.* (1989). Three different assays were used to assess the bioactivity of partially purified samples of CAI. Primarily, BCE rounding activity has been used to guide the purification. Retention of the ability to inhibit DNA synthesis by BCE cells, and to generate avascular zones on the CAM has also been demonstrated throughout the purification.

A major difficulty in the previous purification scheme was the inability of the CAI partially purified by chloroform:methanol extraction to reproducibly bind to and elute from an HPLC column. The finding that CAI was soluble in DMF enabled the use of HPLC for further purification of the molecule. There are several reasons why the DMF extraction facilitated HPLC purification. The DMF extraction could have removed contaminating impurities from the CAI and enabled binding to the C18 column. Alternatively, DMF extraction could have caused a chemical alteration of the CAI molecule, of a carrier, or an associated molecule. This alternative seems less likely, since BCE DNA synthesis inhibition, BCE rounding activity, and CAM angiogenesis inhibitory activity were retained after both the DMF extraction and the HPLC C18 column steps.

Two clearly distinct peaks of rounding activity eluted from both the analytical and semi-preparative C18 HPLC columns (Figures 7-9). Pools of the fractions from the peaks observed on the first analytical column separation were also analyzed for DNA synthesis inhibition and angiogenesis inhibition on the CAM. The material from the later eluting peak of rounding cell activity clearly retained both the ability to inhibit BCE DNA synthesis, and the ability to cause avascular zones in the CAM assay. The early eluting peak from the C18 column was found to have rounding activity, and it inhibited BCE DNA synthesis. This material did not cause avascular zones on the CAM assay, however 4/5 of the embryos died with this treatment, suggesting toxicity. Investigation of the ability to inhibit angiogenesis in the CAM assay has not yet been carried out with material from the C18 semi-preparative column. The first peak of rounding cell activity from this column is better separated from the bulk of UV-absorbing material than with the analytical column. It is possible that the semi-preparative column might provide separation of cell-rounding activity from the material which was toxic on the CAM assay. If isolated from toxic substances, the material from early peak of rounding cell activity may also inhibit angiogenesis on the CAM assay. This early eluting material could represent an alternate form of the CAI. It is possible that the CAI exists as a dimer, or multimer. Alternatively, a carrier molecule could be associated with the CAI molecule.

The second peak of cell rounding activity which eluted from the C18 column also demonstrated inhibition of DNA synthesis, and inhibition of angiogenesis on the CAM, therefore this material was selected for further purification. Because of difficulty in finding another HPLC resin to which the CAI would bind, the ability of CAI to bind to C18 was further exploited. A single semi-preparative column fraction which demonstrated the highest endothelial cell rounding activity (fraction 13) was selected, and run on the C18 analytical column under slightly different solvent conditions. Selection of a single fraction of the CAI activity peak for the next stage of the purification entailed a significant sacrifice of active material. If all active fractions from the HPLC column were recombined, this step involved about a 30% loss of activity from the material loaded onto the column. By selecting only a single active fraction, only 5-10% of this activity was utilized in the next step of purification. Accepting such a significant sacrifice of active material is an obvious disadvantage to the purification scheme. However, this method does yield a high degree of purification which we hoped to be sufficient for structural analysis. The chromatograph obtained from the C18 analytical run of fraction 13 showed few well-delineated peaks (Figure 10). Multiple runs of the C18 analytical column, using fraction 13 from multiple runs of the C18 semi-preparative column, provided identical chromatographs. These peaks were collected as separate fractions for examination of bioactivity. Endothelial cell rounding activity was observed in two of the fractions collected from the column (peaks 1 and 5). Because the

peaks from the analytical column eluted sharply and seemed to represent single species, it was decided that this material might be pure enough for further analysis by mass spectroscopy and that valuable structural information might be obtained from this analysis. There was not enough material accumulated from the C18 analytical column to also establish anti-angiogenic activity on the CAM assay. This work is in progress.

By mass spectroscopy, possible candidates for the active CAI molecule were found at MW 228 and 172. The 228 species was also found in desorptive chemical ionization. Gas exchange was employed to show that this molecule had 3 exchangeable hydrogens (by comparison with NH_3 or ND_3 as the reagent gases), and possible alcohol functions (mass 18 losses). The molecule 2-deoxyuridine fits this description. Therefore this compound was assayed for BCE rounding activity at concentrations up to 10 mM. No rounding activity was observed. However, it is possible that CAI may be co-purified with a carrier or associated molecule which may be required for bioactivity. One approach to test this theory would be to add 2-deoxyuridine to samples of CAI with known activity, and look for enhancement of the bioactivity.

Characterization of the CAI molecule has revealed new information. It is a molecule of very low molecular weight. It is stable to boiling and freezing. It has shown resistance to pronase digestion. Furthermore, sensitive amino acid analysis indicates that it is not a peptide. On the other hand, solubility and

chromatography behaviors of the CAI molecule are consistent with those of a polar lipid. Based on these characteristics, possible candidates for the identity of CAI include sphingolipids, glycolipids, or their metabolites. Recent reports demonstrate that these molecules can perform roles in regulation of vascularization.

Sphingolipids and sphingolipid breakdown products have been shown to have important roles in cell regulation and physiology (Hannun and Bell, 1989). Breakdown products and metabolites of these cellular lipids include arachadonic acid, prostaglandins, leukotrienes eicosanoids, thromboxanes (Needleman *et al.*, 1986; Samuelsson *et al.*, 1987), and diacylglycerol (Nishizuka, 1986). These molecules may function as endogenous modulators of cell function (Hannun and Bell, 1989). Prostaglandins E_1 and E_2 have been demonstrated as nonpeptide angiogenic factors (BenEzra, 1978: Ziche *et al.,* 1982; Form and Auerbach, 1983), and proposed to have a role in rheumatoid arthritis, tumor induced angiogenesis, and lymphocyte induced angiogenesis (Form and Auerbach, 1983). The role of prostaglandins in the eye is not well known; however a recent study of the influence of a fish oil dietary supplement on immunogenic keratitis has looked indirectly at this question (Verby and Van Haeringen, 1990). In this study, rabbits were given either a fish oil diet, or a sunflower seed oil diet, before immune complex keratitis was induced by corneal intrastromal injection of human serum albumin. Fish lipids are known to contain large amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid.

These fatty acids are known to have an effect on prostaglandin and leukotriene synthesis. The sunflower seed diet is rich only in linoleic acid, which does not influence the production of either prostaglandins or leukotrienes. The animals which were given the fish oil diet showed significantly less neovascularization, leukocyte infiltrate, and corneal edema, as compared to the animals fed a sunflower seed oil diet. This study provides a provocative inference that eicosanoids, either prostaglandins or leukotrienes, may play an important role in the inhibition of angiogenesis in the cornea.

An arachadonic acid metabolite produced by the cornea has been recently described as a potent, dose dependent, chemotactic and angiogenic factor (Masferrer *et al.*, 1991). Cytochrome P450 from human and bovine corneal epithelium have been shown to convert arachadonic acid to 12(R)-hydroxy-5,8,14(Z,Z,Z)-eicosatrienoic acid (compound D) (Masferrer *et al.*, 1989) (Murphy *et al.*, 1988). This metabolite has inflammatory properties including stimulation of vasodilation and breakdown of the blood-aqueous barrier (Masferrer *et al.*, 1989). Human, bovine, and rabbit corneas have also been shown to metabolize arachadonic acid to 12(R)-hydroxy-5,8,10,14 eicosatetraenoic acid [12(R)HETE], which is an inhibitor of Na^+-K^+-ATPase (Schwartzman *et al.*, 1987). The actual role of this molecule in the cornea has not been described. Again, given the characteristics of the partially purified CAI, further investigation in the potential relationship to the corneal

cytochrome P450 arachadonic acid metabolites is certainly warranted.

In summary, a reproducible and straightforward method of extraction and partial purification of CAI from bovine cornea has been established. Successful purification of an angiogenesis inhibitor from cornea is of great potential significance. Because it is of low molecular weight and naturally occurring, this compound has the potential to serve as a diffusible, non-antigenic treatment of corneal neovascularization and possibly, of other angiogenic-dependent diseases. Another potential use of purified CAI might be to improve the storage of corneas prior to corneal transplant. It is well known that there is a high incidence of endothelial rejection and graft failure in corneal transplants in which the recipient beds are vascularized is high (Khodadoust, 1973). A storage solution containing added CAI might help tp prevent neovascularization of the transplanted corneas. This exogenously added CAI could diffuse into the cornea and make it less angiogenic. In the current aqueous solutions, endogenous CAI can presumably diffuse out of the stored cornea and into the storage solution, leaving bFGF in Bowman's layer and Descemet's membrane, which can lead to increased neovascularization. Finally, discovery of the structure of this anti-angiogenic compound may help elucidate the mechanism of its role in counteracting angiogenic factors such as bFGF. It is quite possible that the same or very similar compounds may be found in

many other tissues of the body, functioning in the regulation of vascularization.

TABLE 1

DISEASES MANIFESTING PATHOLOGIC ANGIOGENESIS

- Ophthalmology
  - Corneal graft neovascularization
  - Diabetic retinopathy
  - Neovascular glaucoma
  - Trachoma
  - Retrolental fibroplasia
- Internal Medicine
  - Arthritis
  - Scleroderma
- Pediatrics
  - Hemophiliac joints
  - Angiofibroma
  - Hemangioma
- Radiology
  - Arteriovenous malformations
- Orthopedics
  - Nonunion fractures
- Oncology
  - Solid tumors
- Surgery
  - Granulations-burns
  - Vascular adhesions
  - Hypertrophic scars
  - Delayed wound healing
- Dermatology
  - Psoriasis
  - Pyogenic granuloma
- Neurology
  - Osler-Weber Syndrome
- Cardiology
  - Atherosclerotic plaques

TABLE 1 - This table represents a group of diseases previously thought to be unrelated, however, it has been recognized that the principle pathologic feature of each of these conditions is the abnormality of capillary blood vessel growth. Furthermore, there is the possibility that therapeutic control of the abnormal capillary growth could eliminate other manifestations of the disease. (Adapted from Klagsbrun and Folkman, Angiogenesis, Handbook of Experimental Pharmacology. Vol. 95(II), Chapter 37, 1990).

TABLE 2

DISORDERS ASSOCIATED WITH CORNEAL NEOVASCULARIZATION

Atopic keratitis
Sjogren's disease
Acne roseaca
Contact lens overwear
Pterygium
Phylectenulosis
Keratoconjunctivitis
Superior limbic keratitis
Epithelial keratoconjunctivitis
Vitamin A deficiency
- xerophthalmia
- keratomalacia

Lues
Lipid degeneration
Mycobacteria
- T.B.
- leprosy
- fortuitum

Pemphigoid
Mooren's Ulcer
Post-keratoplasty
Radial keratotomy
Alkali burns
Scleritis

Bacterial Ulcers
Herpes simplex virus
Protozoans - acanthamoeba
Fungal ulcers
Trachoma
Kaposi's sarcoma
Sarcoidosis
Lymphocytic hyperplasia
Systemic lupus erythema
Rheumatoid arthritis
Stevens-Johnson syndrome
Wegner's granulomatosis
Polyarteritis nodosa

TABLE 2 - Capillary growth into the normally avascular cornea can result in decreased vision or blindness in the above disease states. Disorders can involve either the corneal epithelium, corneal stroma, or both tissues.

TABLE 3

PURIFIED ANGIOGENIC FACTORS

GROWTH FACTOR	MOLECULAR WEIGHT
Basic FGF	18 000
Acidic FGF	16 500
VEGF	45 000
Angiogenin	14 100
TGF - α	5 500
TGF - β	25 000
TNF - α	17 000
Platelet-derived ECGF	45 000
Angiotropin	4 500
1-Butyryl-glycerol	306

TABLE 3 - These factors have been shown to be angiogenic *in vivo*, and include polypeptide and nonpeptide low molecular weight compounds (Dobson *et al.*, 1990; Rosenthal *et al.*, 1990; Klagsbrun and Folkman, 1990).

TABLE 4

CAI PREVIOUS PURIFICATION METHOD

Extract frozen bovine corneas in 10% ethanol, 24 hrs,4°C

Filter to remove corneas

Re-extract corneas in 10% ethanol, 2 hrs, 4°C, filter

Boil filtrates, 10 min, centrifuge

Evaporate ethanol on Rotovapor, 1 hr

Ultrafiltration of supernatant, 1000MW cut-off filter

Lyophilize filtrate

Extract in 1:1 chloroform:methanol, 18-24 hrs, 25°C, centrifuge

Re-extract pellet in 1:1 chloroform:methanol, 2 hrs, 25°C, centrifuge

Combine supernatants from chloroform:methanol extractions

Dry on RotoVapor

TABLE 4 - A method of extraction and partial purification of CAI from bovine cornea established by Susan Doctrow and Ed Mun is presented above.

TABLE 5

CAI CURRENT PURIFICATION METHOD

Extract frozen bovine corneas in 100°C distilled H_2O, 20 min

Filer to remove corneas, centrifuge filtrate

Ultrafiltration of supernatant, 1000MW cut-off filter

Lyophilize filtrate

Extract in 90% dimethylformamide (DMF), 1hr, 25°C

Centrifuge, dry supernatant on RotoVapor

100% DMF extraction, 1 hr, 25°C

Dry extract in Speed Vac

HPLC C18 Semi-preparative column
Elute with 0 - 100% acetonitrile, 0.1% trifluoroacetic acid

HPLC C18 Analytic column
Elute with 0 - 50% acetonitrile

TABLE 5 - This schematic represents the current CAI partial purification method. This method has demonstrated improvement in reproducibility, and more significantly, DMF extraction of CAI has allowed for consistent utilization of HPLC to obtain further purification.

TABLE 6

ACTIVTY YIELDS OF THE CURRENT CAI PURIFICATION

Boiling Extraction	100%
Ultrafiltration	80%
90% DMF Extraction	90%
100% DMF Extraction	85%
Semi-preparative C18 HPLC - all active fractions totaled	70%
Single fraction (13) - selected for further HPLC purification	5-10%
C18 analytical HPLC column - total of active fractions	80%

TABLE 6 - The above table presents the approximate yields of activity of each step in the purification scheme. Activity is based on observed bioactivity in the semi-quantitative BCE rounding cell assay. Activities were calculated as the % of activity retrieved from the total activity of the starting material from the previous step.

TABLE 7

ANTI-ANGIOGENIC ACTIVITY OF C18 HPLC FRACTIONS

CHICK CHORIOALLANTOIC MEMBRANE ASSAY

C18 Fractions	Rounding Units	% Avascular Zones	Observations
1 - 4*	126	0 %	Toxic to 4/5 eggs
5 - 9*	81	20 %	1/5 small zone
10 - 13*	114	100 %	5/5 large avascular zones
14 - 16*	18	40 %	2/5 small zones

* Fractions pooled from 6 runs of C18 HPLC analytical column

TABLE 7 - Fractions collected from a sample of 100% DMF extracted CAI material run on a C18 analytic HPLC column (**Figure 7**) were pooled into four groups: Fractions 1-4, 5-9, 10-13, 14-16. The samples were assayed for BCE rounding activity and angiogenesis inhibition on the CAM. The pool of fraction 10-13 showed dramatic anti-angiogenic activity, and high BCE rounding activity.

FIGURE 1

SCHEMATIC REPRESENTATION
OF THE VASCULAR AND AVASCULAR STRUTURES
OF THE BOVINE AND HUMAN EYE

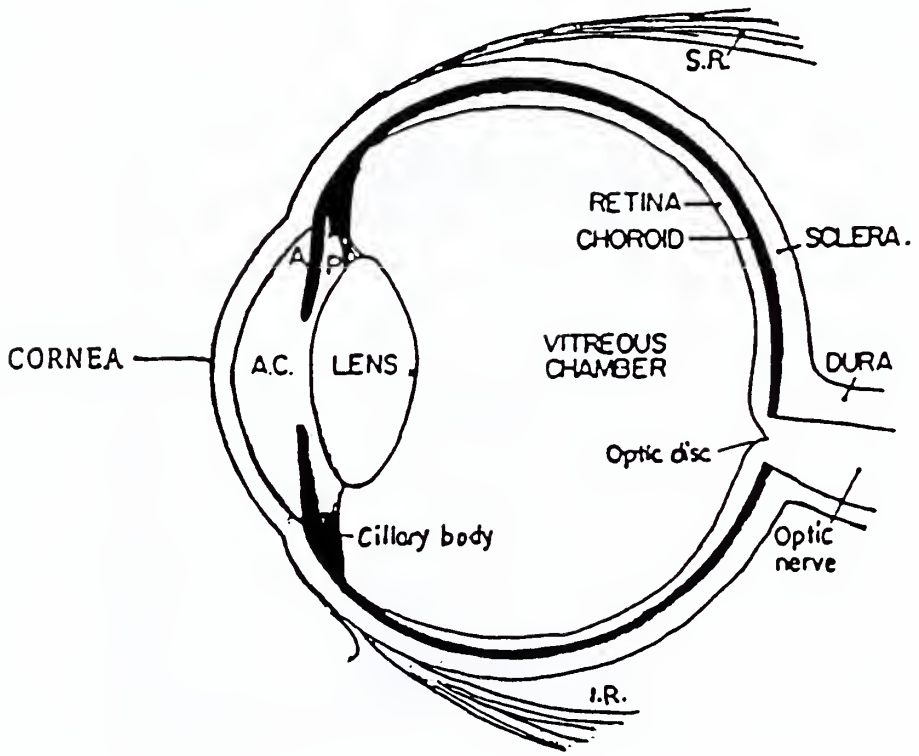

FIGURE 1 - The eye is divided into vascular and avascular zones. The cornea, lens, sclera, aqueous humor and vitreous humor are all normally avascular zones. Infringement of blood vessel growth into these tissues can lead to decreased vision or blindness (Diagram adapted from Goldberg, 1984).

FIGURE 2

SCHEMATIC REPRESENTATION OF HISTOLOGIC SECTIONS OF THE CORNEA

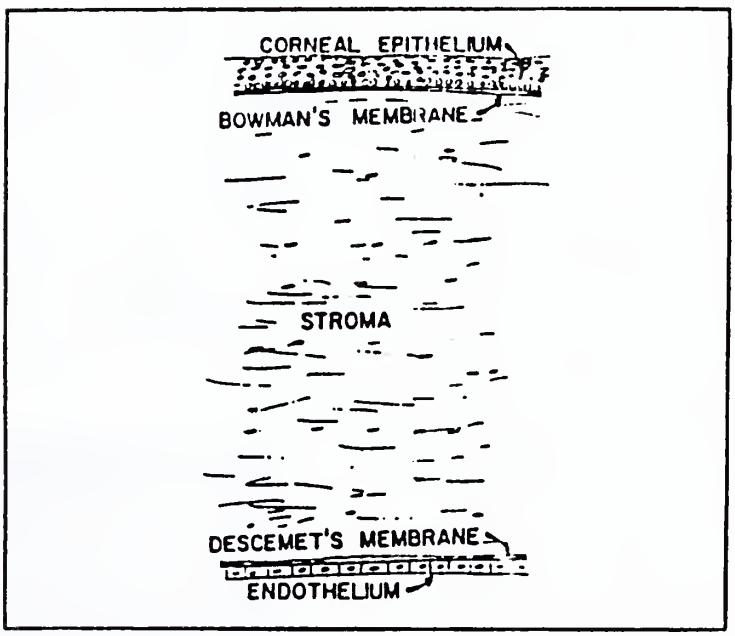

FIGURE 2 - Bowman's layer is the acellular, anterior-most portion of the collagenous corneal stroma, rich in heparan-sulfate proteoglygan. Descemet's membrane is a true basement membrane produced by corneal endothelial cells on the posterior surface of the cornea. Both layers bind bFGF (Adamis *et al.*, 1991; Diagram adapted from Goldberg, 1984).

FIGURE 3

ENDOTHELIAL CELL ROUNDING ASSAY

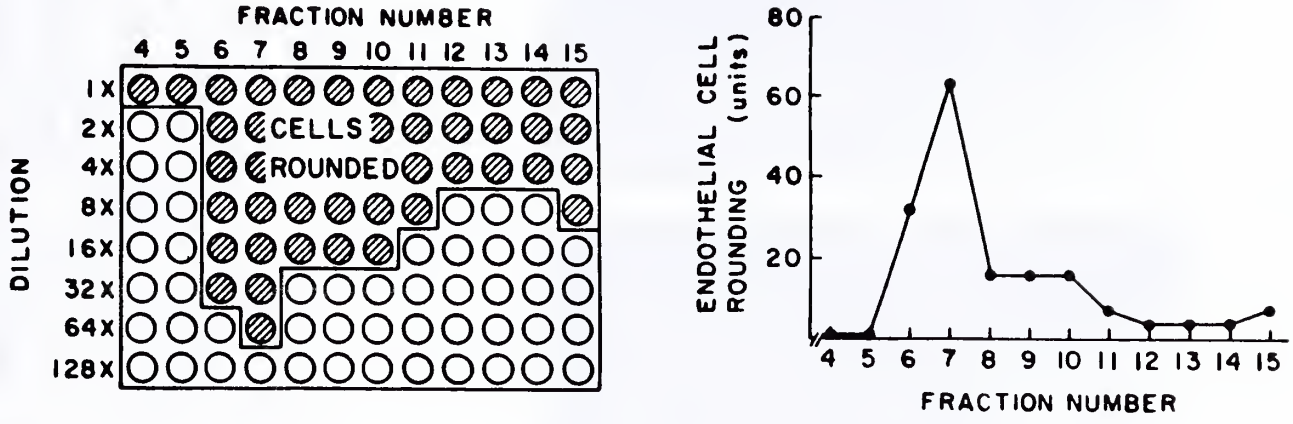

FIGURE 3 - The units of CAI activity in a given sample are based on the ability of the sample to cause endothelial cell rounding. For this assay, BCE cells were seeded onto 96 well 1/2 area plates at density of 2000 cells/25µl/well. After a 12 h attachment period, cells were treated with varied concentrations of inhibitor fractions, by making two-fold serial dilutions vertically, as indicated in the sample diagram above. After 24 hours , the cells were scored as either positive (shaded) or negative (open) for cell rounding. A positive sample is one in which at least 50% of the cells in the well are rounded (see **Figure 4**). One unit of cell rounding is defined as the amount of sample sufficient to cause rounding in only 50% of the cells in a given well. The number of rounding units in a given sample is calculated from the greatest dilution of that sample which will produce one unit of cell rounding. A graphic profile of rounding units calculated for the fractions in this sample assay is also shown. (Adapted from Doctrow and Mun, unpubl.)

FIGURE 4

CAI TREATED BOVINE CAPILLARY ENDOTHELIAL CELLS

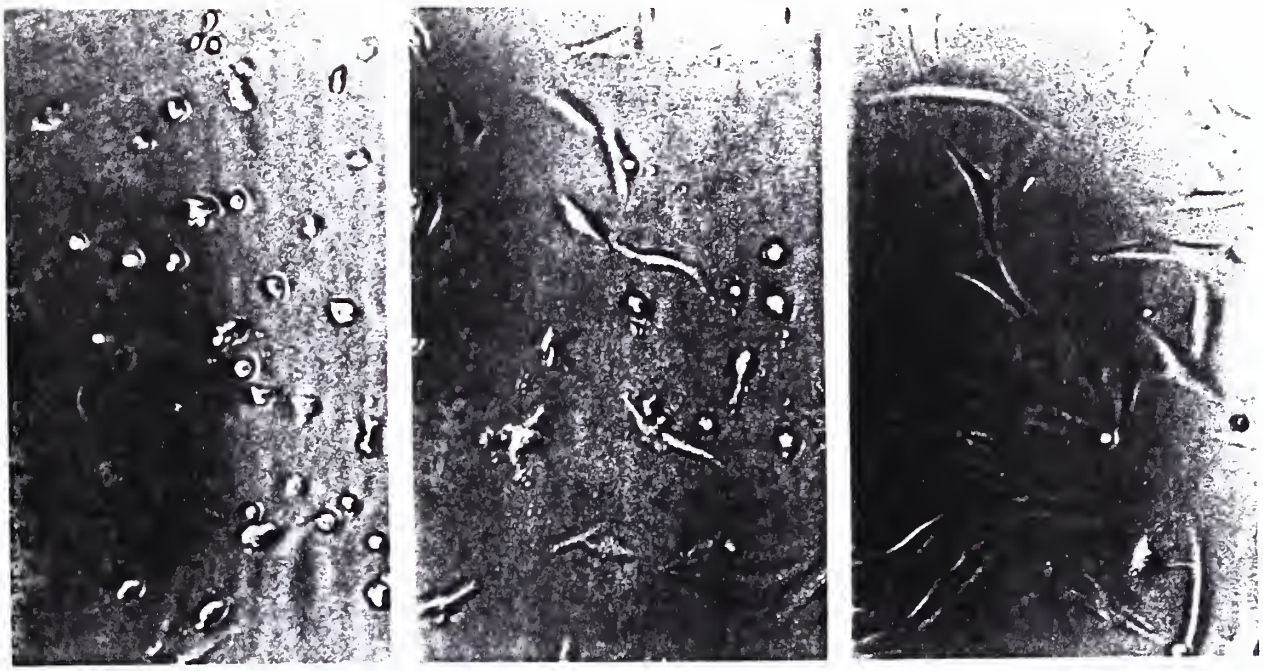

FIGURE 4 - Bovine capillary endothelial cells were plated at a density of 2000 cells per 25 µl per well, in 96 well 1/2 area plates. These cells were treated with serial dilutions of CAI inhibitor samples (decreasing concentrations from left to right). The inhibitor causes rounding of the cells after 10 - 24 hours, in a concentration dependent manner. One unit of cell rounding as defined as the amount of sample sufficient to cause rounding of 50% of the cells in a given well. The cells in the middle well above would be scored as 50% round. The rounded cells exclude trypan blue and stain with the viability marker fluorescein diacetate

FIGURE 5

CAI INHIBITION OF ANGIOGENESIS ON THE CHICK EMBRYO CHORIOALLANTOIC MEMBRANE

FIGURE 5 - Application of a sample of partially purified CAI to the chorioallantoic membrane of a six-day chick embryo causes capillary regression and produces a 4 mm avascular zone at 48 hrs, indicating inhibitory activity. The chick embryo was injected with India ink prior to dissection to improve visualization of the vessels. (Photo courtesy of Susan Doctrow and Ed Mun).

FIGURE 6

BOVINE CORNEA DISSECTION

FIGURE 6 - Bovine eyes (-70°C) were rinsed briefly in cold distilled H_20, and the cornea carefully excised from the frozen eye using a number 15 scalpel blade. The corneas were placed on dry ice and stored at -80°C.

FIGURE 7

C18 ANALYTIC HPLC COLUMN
100% DMF EXTRACTED CAI SAMPLE

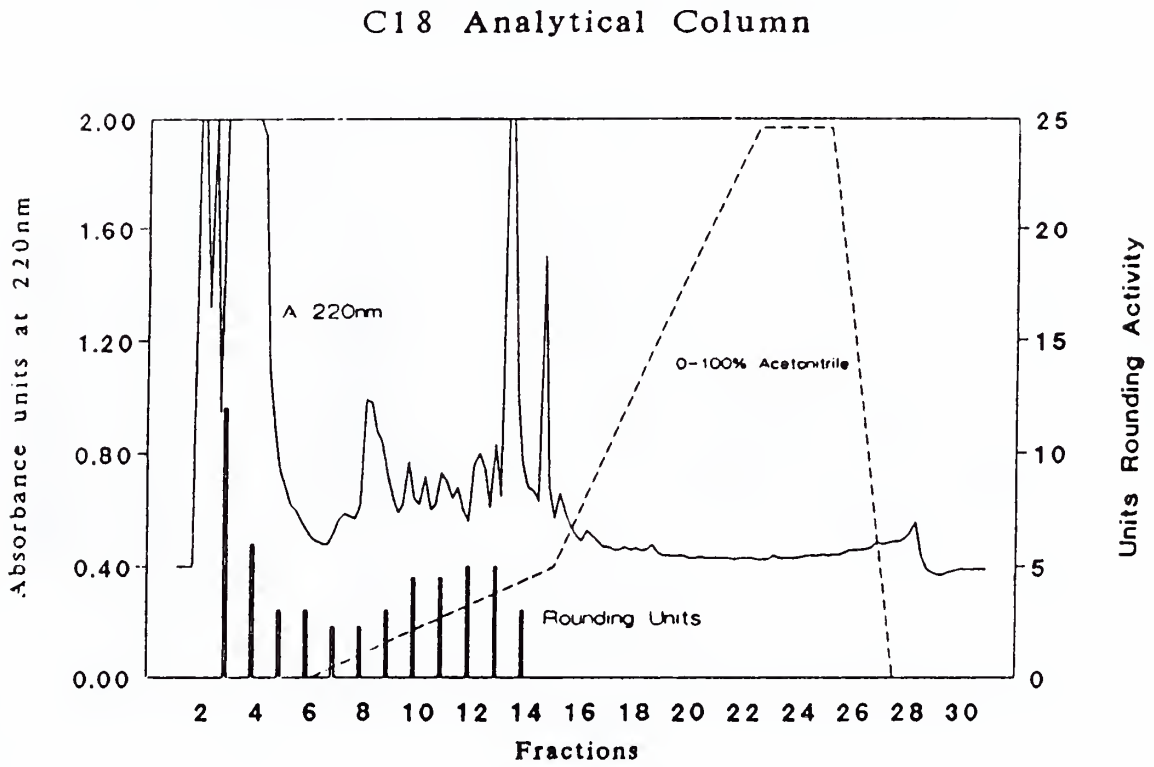

FIGURE 7 - This chromatograph was obtained with a sample of CAI which had been extracted in 100% DMF. CAI rounding cell activity was found to consistently bind and elute from the C18 analytic column as shown above. Two major peaks of rounding cell activity were observed, and fractions from both peaks also inhibited BCE DNA synthesis. Only the second peak, however, was shown to have angiogenic inhibitory capability on the CAM assay.

FIGURE 8

C18 SEMIPREPARATIVE HPLC COLUMN
100% DMF EXTRACTED CAI SAMPLE

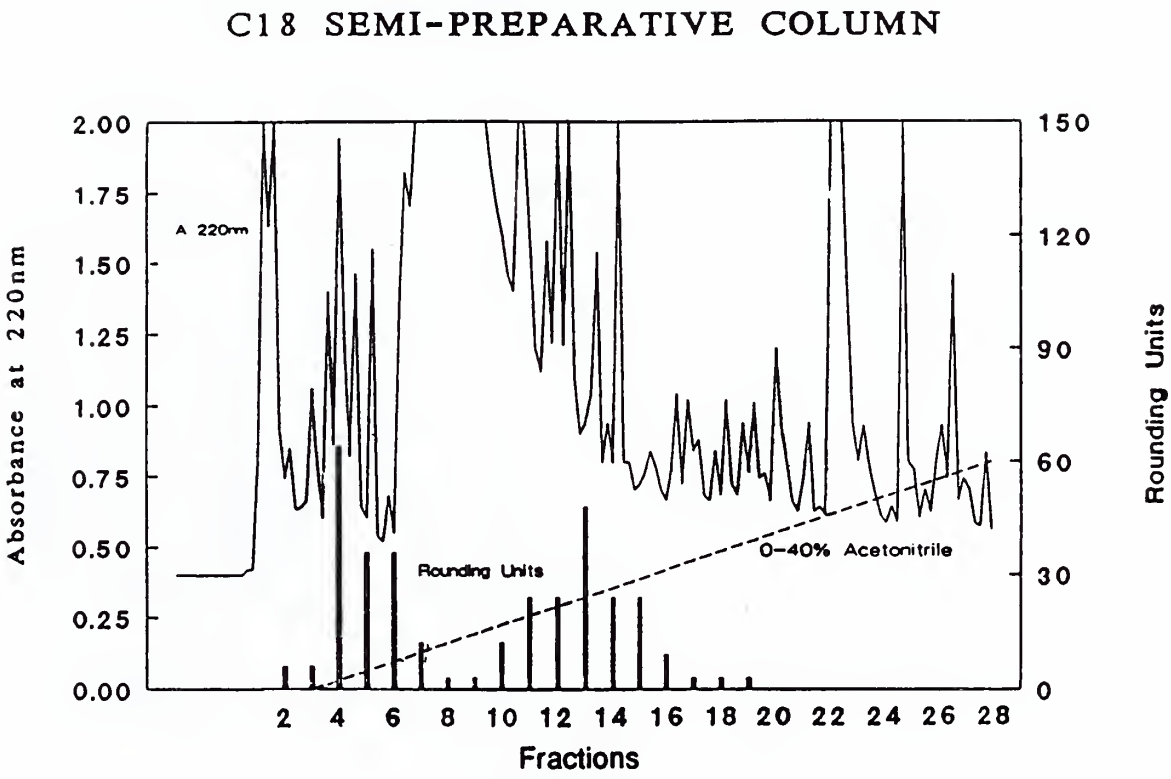

FIGURE 8 - In order to increase the amount of CAI purified using C18 HPLC, a semipreparative C18 HPLC column was employed. Using the larger column, better resolution and separation of CAI bioactivity was obtained. As with the analytical column, two major peaks of BCE rounding activity were observed. Notably, fractions 3 and 4 collected from this column were highly acidic, but contained no rounding activity.

FIGURE 9

C18 ANALYTICAL HPLC COLUMN
SECOND HPLC PURIFICATION STEP

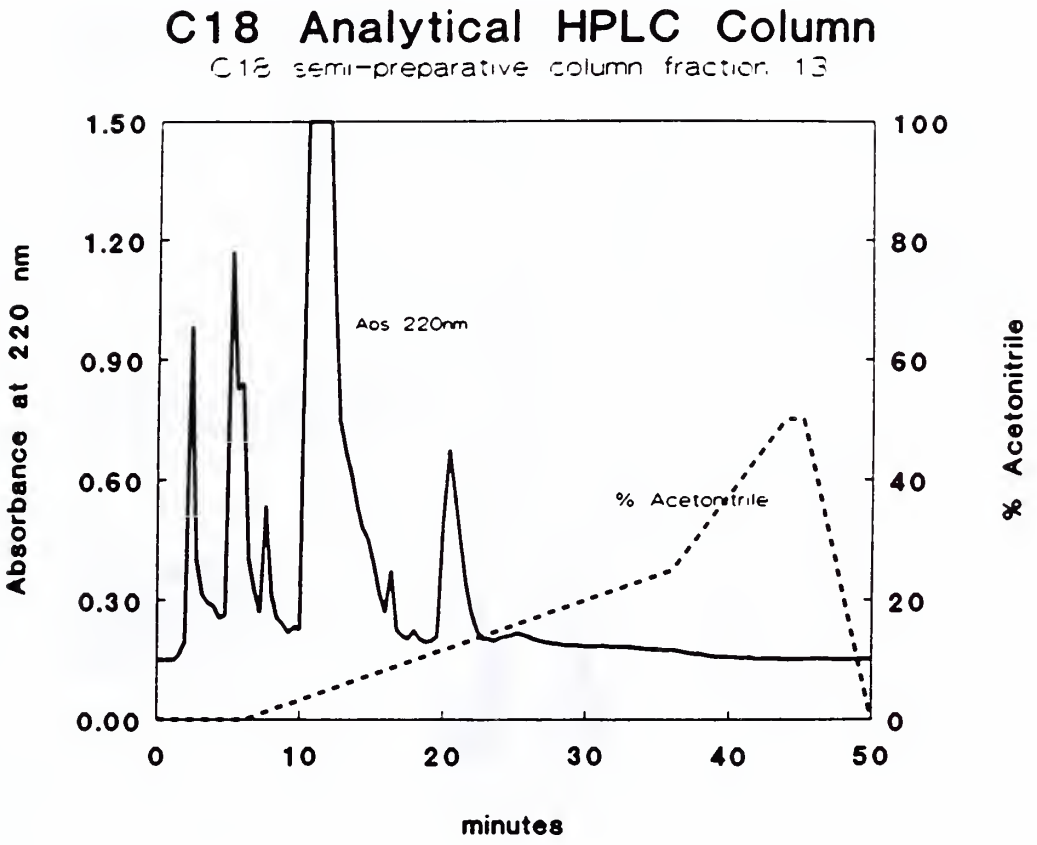

FIGURE 9 - To further the purification of CAI, a single fraction from the C18 semi-preparative column (Fraction 13) which contained high BCE rounding activity (**Figure 8**) was selected, and run on a C18 analytical column, with a 0 - 50% gradient of acetonitrile (no TFA). The above chromatograph was obtained, and the individual peaks collected as separate fractions and assayed for bioactivity.

FIGURE 10

C18 ANALYTICAL HPLC COLUMN BIOACTIVITIES AND WEIGHTS OF SAMPLES SUBMITTED FOR MASS SPECTROSCOPY

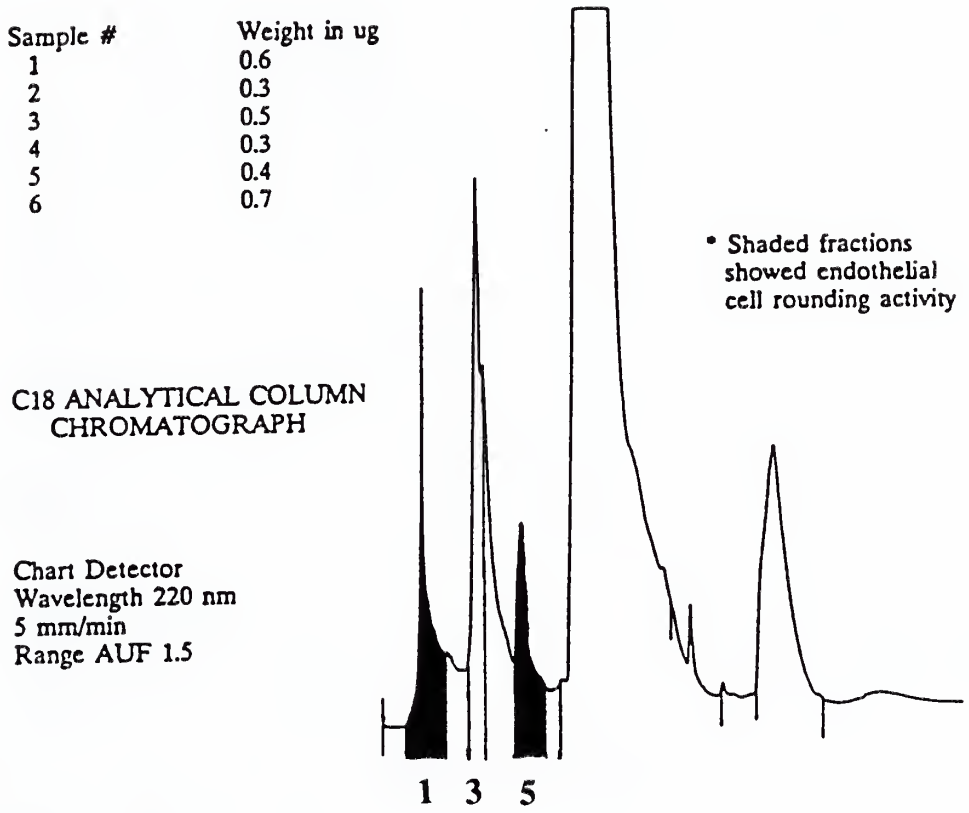

FIGURE 10 - Fractions were collected as marked on the chromatograph (1 - 5), and assayed for rounding activity. Fraction 1 contained 7 units of rounding activity, fraction 5 contained 6 units.

Another run was performed under identical conditions, and these same fractions were collected in baked glass tubes, dried in the SpeedVac, and weighed. These samples were submitted for mass spectroscopy, with samples 1 and 5 corresponding to active fractions.

FIGURE 11

EFFECT OF CAI ON BCE DNA SYNTHESIS

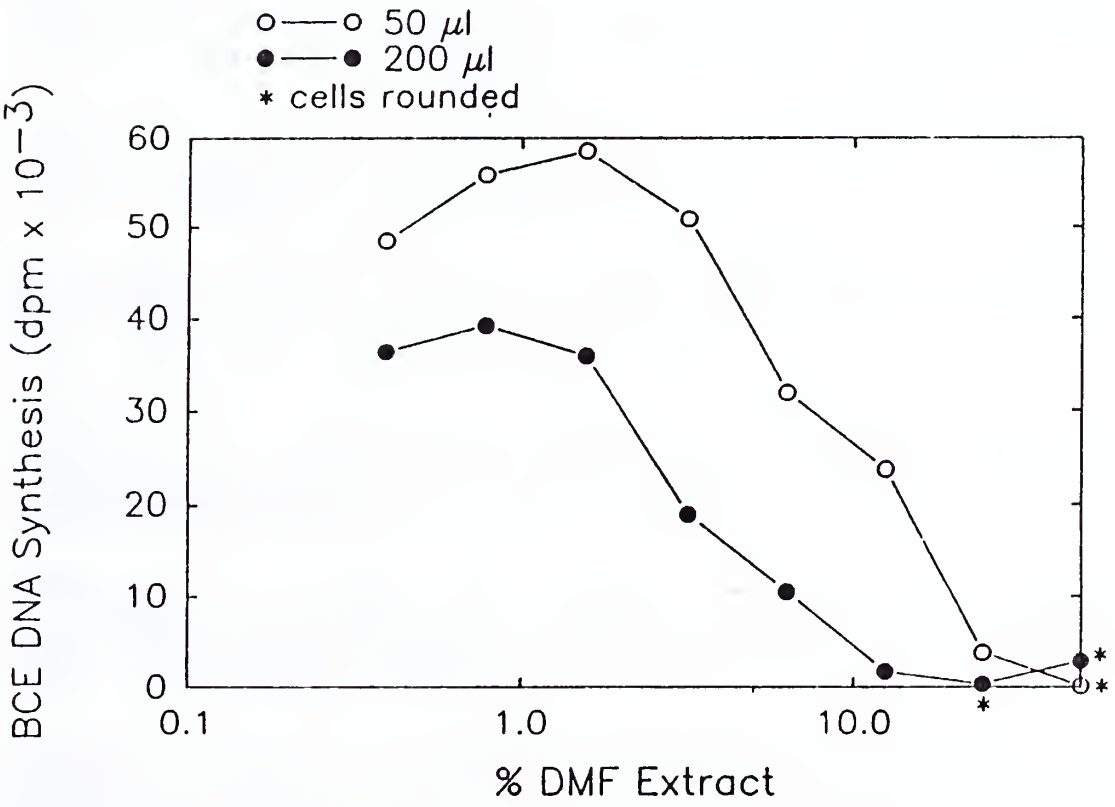

FIGURE 11 - CAI partially purified by DMF extraction which had demonstrated bioactivity in the BCE rounding assay was assayed for bioactivity in a DNA synthesis experiment. The DMF extracted CAI sample demonstrated significant inhibition of DNA synthesis, at concentrations of CAI much less than required for rounding activity. Similar results had been obtained with the chloroform:methaol extracted material from the previous purification scheme.

REFERENCES

Adamis, A.P., B. Meklir, and N.C. Joyce. "In Situ Injury-Induced Release of Basic-Fibroblast Growth Factor from Corneal Epithelial Cells." American Journal of Pathology 139 (1991): 961-967.

Aiello, L.M., M. Wand, and G. Liang. "Neovascular Glaucoma and Vitreous Hemorrhage Following Cataract Surgery in Patients with Diabetes Mellitus." Opthalmology 90 (1983): 814-820.

Ashton, N. "Retinal Vascularization in Health and Disease." American Journal of Opthalmology 44 (1957): 7-17.

Ashton, M. "Pathogenesis of Diabetic Retinopathy." In Diabetic Retinopathy, ed. H.L. Little, R.L. Jack, A. Patz, and P.H. Forsham. 85-97. New York: Thieme-Stratton, 1983.

Auerbach, R., L. Kubai, D. Knighton, and J. Folkman. "A Simple Procedure for the Long-Term Cultivation of Chicken Embryos." Developmental Biology 41 (1974): 391-394.

Auerbach, R., L. Alby, L.W. Morrissey, M. Tu, and J. Joseph. "Expression of Organ-Specific Antigens on Capillary Endothelial Cells." Microvascular Research 29 (1985): 401-411.

Ausprunk, D.H. and J. Folkman. "Migration and Proliferation of Endothelial Cells in Preformed and Newly formed Blood Vessels During Tumor Angiogenesis." Microvascular Research 14 (1977): 53-65.

Bagavandoss, P. and J.W. Wilks. "Specific Inhibition of Endothelial Cell Proliferation by Thrombospondin." Biochemical and Biophysical Research Communications 170 (1990): 867-872.

Baird, A., F. Esch, P. Bohlen, N. Ling, and D. Gospodarowicz. "Isolation and Partial Characterization of an Endothelial Cell Growth Factor from the Bovine Kidney." Regulation of Peptides 12 (1985): 201.

Baird, A., P. Mormede, and P. Bohlen. "Immunoreactive Fibroblast Growth Factor In Cells of Peritoneal Exudate Suggests its

Identity with Macrophage-Derived Growth Factor." Biochemical and Biophysical Research Communications 126 (1985): 358-364.

Baird, A., F. Esch, D. Gospodarowicz, and R. Guillemin. "Retinal Derived Endothelial Cell Growth Factors: Partial Molecular Characterization and Identity with Acidic and Basic Fibroblast Growth Factor." Biochemistry 24 (1985): 7859-7878.

Baird, A. and T. Durkin. "Inhibition of Endothelial Cell Proliferation by Type B-Transforming Growth Factor: Interactions with Acidic and Basic Fibroblast Growth Factors." Biochemical and Biophysical Research Communications 138 (1986): 476-482.

Barger, A.C., R. Beeuwkes III, L.L. Lainet, and K.J. Silverman. " Hypothesis: Vasa Vasorum and Neovascularization of Human Coronary Arteries." New England Journal of Medicine 316 (1984): 175-177.

Baudin, C., D. Fredj-Reygrobellet, J. Caruelle, D. Barritault, P. Gastaud, and P. Lapalus. "Acidic Fibroblast Growth Factor Distribution in Normal Human Eye and Possible Implications in Ocular Pathogenesis." Opthlamic Research 22 (1990): 73-81.

Bär, T. Advances in Anatomy, Embryology and Cell Biology. ed. A. Brodal, W. Hild, and J. Van Limborgh. Berlin and New York: Springer-Verlag, 1980.

Ben Ezra, D. "Neovasculogenic Ability of Prostaglandins, Growth Factors and Synthetic Chemoattractants." American Journal of Opthalmology 86 (1978): 455-461.

Bohlen, P, A. Baird, F. Esch, N. Ling, and D. Gospodarowicz. "Isolation and Partial Molecular Characterization of Pituitary Fibroblast Growth Factor." Proceedings of the National Academy of Science 81 (1984): 5364-5368.

Brem, H. and J. Folkman. "Inhibition of Tumor Angiogenesis." Experimental Medicine 141 (1975): 427-439.

Brem, S., H. Brem, J. Folkman, D. Finkelstein, and A. Patz. "Prolonged Tumor Dormancy by Prevention of Neovascularization in Vitreous." Cancer Research 1976 (1976): 2807-2812.

Conn, G. and V.B. Hatcher. "The Isolation and Purification of Two Anionic Endothelial Cell Growth Factors from Human Brain." Biochemical and Biophysical Research Communications 124 (1984): 262-268.

Connolly, D.T., D.M. Heuvelman, R. Nelson, J.V. Olander, V.L. Eppley, J.J. Delfino, M.R. Siegel, R.M. Leimgruber, and J. Feder. "Tumor Vascular Permeability Factor Stimulates Endothelial Cell Growth and Angiogenesis." Journal of Clinical Investigation 84 (1989): 1470-1478.

Courty, J., C. Loret, M. Moenner, B. Chevallier, O. Lagente, Y. Courtois, and Barritault D. "Bovine Retina Contains Three Growth Factor Activities with Different Affinity for Heparin: Eye-Derived Growth Factor I, II and III." Biochimie 67 (1985): 265.

Crisp, A., C.M. Chapman, S.E. Kirkham, A.L. Schiller, and S.M. Krane. "Articular Mastrocytosis in Rheumatoid Arthritis." Arthritis and Rheumatology 27 (1984): 845-851.

Crum, R., S. Szabo, and J. Folkman. "A New Class of Steroids Inhibits Angiogenesis in the Presence of Heparin or Heparin Fragment." Science 230 (1985): 1375-1378.

D'Amore, P.A. and M. Klagsbrun. "Endothelial Cell Mitogens Derived From Retina and Hypothalamus: Biochemical and Biological Similarities." Journal of Cell Biology 99 (1984): 1545-1549.

Denekamp, J. "Vasculature as a Target for Tumor Therapy." In Progress in Applied Microcirculation, ed. F. Hammerson and O. Hudlicka. 28-38. Basel: Karger, 1984.

Dobson, D.E., A. Kambe, E. Block, T. Dion, H. Lu, J.J. Castellot, and B.M. Spiegelman. "1-Butyryl-Glycerol: A Novel Angiogenesis Factor Secreted by Differentiating Adipocytes." Cell 61 (1990):

Eisenstein, R., N. Sorgente, L. Soble, A. Miller, and K.E. Kuettner. "The Resistance of Certain Tissues to Invasion: Penetrability of Explanted Tissues by Vacularized Mesenchyme." American Journal of Pathology 73 (1973): 765-774.

Engerman, R.L., D. Pfaffenbach, and M.D. Davis. "Cell Turnover of Capillaries." Lab Investigation 17 (1967): 738-743.

Esch, F., A. Baird, N Ling, N. Ueno, F. Hill, L. Denoroy, R. Klepper, D. Gospodarowicz, P. Bohlen, and R. Guillemin. "Primary Structure of Bovine Pituitary Basic Fibroblast Growth Factor (FGF) and Comparison with the Amino-Terminal Sequence of Bovine Acidic FGF." Proceedings of the National Academy of Science USA 82 (1985): 6507-6511.

Ferrara, N. and W.J. Henzel. "Pituitary Follicular Cells Secrete a Novel Heparin-Binding Growth Factor Specific for Vascular Endothelial Cells." Biochemical and Biophysical Research Communications 161 (1989): 851-858.

Ferrara, N., C. Clapp, and R. Weiner. "The 16K Fragment of Prolactin Specifically Inhibits Basal or Fibroblast Growth Factor Stimulated Growth of Capillary Endothelial Cells." Endocrinology 129 (1991): 896-900.

Folkman,J. "Towards a new understanding of vascular proliferative disease in children." Pediatrics 74 (1984) 850-856).

Folkman, J.F., C.C. Haudenschild, and B.R. Zetter. "Long-Term Culture of Capillary Endothelial Cells." Proceedings of the National Academy of Science USA 76 (1979): 5217-5221.

Folkman, J. and Haudenschild. "Angiogenesis In Vitro." Nature 288 (1980): 551-556.

Folkman, J. "Tumor Angiogenesis." Advances in Cancer Research 43 (1985): 175-203.

Folkman, J. and M Klagsbrun. "Angiogenic Factors." Science 235 (1987): 442-227.

Folkman, J., M. Klagsbrun, J. Sasse, D. Ingber, M. Wadzinski, and I. Vlodavsky. "A Heparin-Binding Angiogenic Protein - Basic Fibroblast Growth Factor - Is Stored Within Basement Membrane." American Journal of Pathology 130 (1988): 393-400.

Folkman, J. "Evidence that Tumors are Angiogenesis Dependent." Journal of National Cancer Institute 82 (1990): 4-6.

Folkman, J., S. Szabo, M. Stovroff, P. McNeil, Li W., and Y. Shing. "Duodenal Ulcer." Annals of Surgery (1991):

Form, D.M. and R. Auerbach. "PGE2 and Angiogenesis." Proceedings of the Society for Experimental Biology and Medicine 172 (1983): 214-218.

Frater-Schroder, M., W. Risau, R. Hallman, R. Gautschi, and P. Bohlen. "Tumor Necrosis Factor Type-alpha, a Potent Inhibitor of Endothelial Cell Growth In Vitro, Is Angiogenic In Vivo." Proceedings of the National Academy of Science USA 84 (1987): 5277-5281.

Fromer, C.H. and G.K. Klintworth. " An Evaluation of the Role of Leukocytes in the Pathogenesis of Experimentally Induced Corneal Vascularization." American Journal of Pathology 79 (1975): 537-550.

Fromer, C.H. and G.K. Klintworth. "An Evaluation of the Role of Leukocytes in the Pathogenesis of Experimentally Induced Corneal Vascularization III. Studies Related to the Vasoproliferative Capability of Polymorphonuclear Leukocytes and Lymphocytes." American Journal of Pathology 82 (1976): 157-167.

Garner, A. "Ocular Angiogenesis." International Journal of Experimental Pathology 28 (1986): 249-309.

Gitlin, J.D. and P.A. D'Amore. "Culture of Retinal Capillary Endothelial Cells Using Selective Growth Media." Microvascular Research 26 (1983): 74-80.

Goldberg, S. Clinical Anatomy Made Ridiculously Simple. Miami, Florida: MedMaster, Inc., 1984.

Good, D.J., P.J. Polerini, F. Rastinejad, M.M. Le Beau, R.S. Lemons, W.A. Frazier, and N.P. Bouck. "A Tumor-Suppressor Dependent Inhibitor of Angiogenesis is Immunologically and Functionally Indistinguishable from a Fragment of Thrombospondin." Proceedings of the National Academy of Science 87 (1990): 6624-6628.

Gospodarowicz, D. "Localization of A Fibroblast Growth Factor and its Effect Alone and with Hydrocortisone on 3T3 Cell Growth." Nature 249 (1974): 123-129.

Gospodarowicz, D. "Purification of a Fibroblast Growth Factor from Bovine Pituitary." Journal of Biological Chemistry 250 (1975): 2515-2520.

Gospodarowicz, D. and K. Thakral. "Production of a Corpus Luteum Angiogenic Factor Responsible for Proliferation of Capillaries and Neovascularization of the Corpus Luteum." Proceedings of the National Academy of Science USA 75 (1978): 847-851.

Gospodarowicz, D., J. Cheng, G.M. Lui, A Baird, and P. Bohlen. "Isolation by Heparin-Sepharose Affinity Chromatography of Brain Fibroblast Growth Factor: Identity with Pituitary Fibroblast Growth Factor." Proceedings of the National Academy of Science USA 81 (1984): 6963-6967.

Gospodarowicz, D., J. Cheng, G. Lui, A. Baird, F. Esch, and P. Bohlen. "Corpus Luteum Angiogenesis Factor is Related to Fibroblast Growth Factor." Endocrinology 117 (1985): 2283-2391.

Gospodarowicz, D., A. Baird, J. Cheng, G.M. Lui, F. Esch, P. Bohlen. "Isolation of Basic Fibroblast Growth Factor from Bovine Adrenal Gland: Psyiochemical and Biological Characterization." Endocrinology 118 (1986): 82-90.

Gospodarowicz, D., J.A. Abraham, and J. Schilling. "Isolation and Characterization of a Vascular Endothelial Cell Mitogen Produced by Pituitary-Derived Folliculostellate Cells." Proceedings of the National Academy of Science USA 86 (1989): 7311-7315.

Grant, M., J. Jerdan, and T.J. Merimee. "Insulin-Like Growth Factor-I Modulates Endothelial Cell Chemotaxis." Journal of Clinical Endocrinology and Metabolism 65 (1987): 370-371.

Gross, J.L., D. Moscatelli, and D.B. Rifkin. "Increased Capillary Endothelial Cell Protease Activity in Response to Angiogenic Stimuli in vitro." Proceedings of the National Academy of Science USA 80 (1983): 2623-2627.

Hannun, Y.A. and R.M. Bell. "Functions of Sphingolipids and Sphingolipid Breakdown Products in Cellular Regulation." Science 243 (1989): 500-507.

Haudenschild, C.C. Growth Control of Endothelial Cells in Atherogenesis and Tumor Angiogenesis. Vol. 9. Advances in Microcirculation, ed. B.M. Altura, E. Davis, and H. Harders. Basel: Karger, 1980.

Hauschka, P.V., A.E. Mavrokos, M. Iafrati, S.E. Doleman, and M. Klagsbrun. "Growth Factors in Bone Matrix: Isolation of Multiple Types by Affinity Chromatography on Heparin-Sepharose." Journal of Biological Chemistry 261 (1986): 12665-12674.

Hertig, A.T. Contributions to Embryology 25 (1935): 37.

Hunt, T.K. Wound Healing and Wound Infection: Theory and Surgical Practice. New York: Appelton-Century-Crofts, 1980.

Hunt, T.K., W.S. Andrews, B. Halliday, G. Greenberg, D. Knighton, R. Clark, and K. Thakral. The Surgical Wound. ed. P. Dineen and G. Hildick-Smith. Philidelphia, Pennsylvania: Lea & Febiger, 1981.

Ingber, D.E., J.A. Madri, J. Folkman. "A Possible Mechanism for Inhibition of Angiogenesis by Angiostatic Steroids: Induction of Capillary Basement Membrane Dissolution." Endocrinology 119 (1986): 1768-1775.

Jakob, W., K.D. Jentzsch, B. Mauersberger, and P. Oehme. "Demonstration of Angiogenesis Activity in the Corpus Luteum of Cattle." Experimental Pathology 13 (1977): 231-236.

Jennings, J.C., S. Mohan, T.A. Linkhart, R. Widstrom, and D.J. Baylink. "Comparison of the Biological Actions of TGF-Beta-1 and TGF-Beta-2: Differential Activity In Endothelial Cells." Journal of Cell Physiology 137 (1988): 167-172.

Kaufman, H.E., R.W. Beuerman, T.L. Steinemann, H.W. Thompson, and E.D. Varnell. "Optisol Corneal Storage Medium." Archives of Opthalmology 109 (1991): 864-868.

Khodadoust, A.A. "The Allograft Rejection: The Leading Cause of Late Failure of Clinical Corneal Grafts." Ciba Foundation Symposium (1973), 151-164.

King, G.L., A.D. Goodman, S. Buzney, A. Moses, and C.R. Kahn. "Receptors and Growth Promoting Effects of Insulin Like Growth Factors on Cells from Bovine Retinal Capillaries and Aorta." Journal of Clinical Investigation 75 (1985): 1028-1036.

Klagsbrun, M., D. Knighton, and J. Folkman. "Tumor Angiogenesis in Cells Grown in Tissue Culture." Cancer Research 36 (1976): 110-114.

Klagsbrun, M. and Y. Shing. "Heparin Affinity of Anionic and Cationic Capillary Endothelial Cell Growth Factors: Analysis of Hypothalamus-Derived Growth Factors and Fibroblast Growth Factors." Proceedings of the National Academy of Science USA 82 (1985): 805.

Klagsbrun, M., J. Sasse, R. Sullivan, and J.A. Smith. "Human Tumor Cells Synthesize an Endothelial Cell Growth Factor that is Structurally Related to Basic Fibroblast Growth Factor." Proceedings of the National Academy of Science USA 83 (1986): 2448-2452.

Klagsbrun, M. and J. Folkman. Angiogenesis. Vol. 95/II. Handbook of Experimental Pharmacology, Peptide Growth Factors and their Receptors, ed. M.B. Sporn and A.B. Roberts. Heidelberg: Springer-Verlag Berlin, 1990.

Koch, A.E., P.J. Polverini, and S.J. Leibovich. "Stimulation of Neovascularization by Human Rheumatoid Synovial Tissue Macrophages." Arthritis and Rheumatology 29 (1986): 471-479.

Lemmon, S.K. and R. Bradshaw. "Purification and Partial Characterization of Bovine Pituitary Fibroblast Growth Factor." Journal of Cell Biochemistry 21 (1983): 195-208.

Lobb, R.R. and J.W. Fett. "Purification of Two Distinct Growth Factors from Bovine Neural Tissue by Heparin Affinity Chromatography." Biochemistry 23 (1984): 6295-6299.

Lutty, G.A., R.J. Mello, C. Chandler, C. Fait, A. Bennet, and A. Patz. "Regulation of Cell Growth by Vitreous Humor." Journal of Cell Science 76 (1985): 53-65.

Maciag, T., T. Mehlman, R. Friesel, and A.B. Schreiber. "Heparin binds Endothelial Cell Growth Factor, the Principal Cell Mitogen in Bovine Brain." Science 225 (1984): 932.

Maione, T.E., G.S. Gray, J. Petro, A.J. Hunt, A.L. Donner, S.I. Bauer, H.F. Carson, and R.J. Sharpe. "Inhibition of Angiogenesis by Recombinant Human Platelet Factor-4 and Related Peptides." Science 247 (1990): 77-79.

Masferrer, J.L., R.C. Murphy, P.J. Pagano, M.W. Dunn, and M. Laniado-Schwartzman. "Ocular Effects of a Novel Cytochrome P-450-Dependent Arachidonic Acid Metabolite." Investigative Opthalmology & Visual Science 30 (3 1989): 454-460.

Masferrer, J.L., J.A. Rimarachin, M.E. Gerritsen, J.R. Falck, P. Yadagiri, M.W. Dunn, and M. Laniado-Schwartzman. "12(R)-Hydroxyeicosatrienoic Acid, A Potent Chemotactic and Angiogenic Factor Produced by the Cornea." Experimental Eye Research 52 (1991): 417-424.

Matsubara, T. and M. Ziff. "Inhibition of Human Endothelial Cell Proliferation by Gold Compounds." Journal of Clinical Investigation 79 (1987): 1440-1446.

McIntosh, L.C., J.C. Gaal, and J.V. Forrester. "Serum-Induced Proliferation of Retinal Capillary Endothelial Cells is Inhibited in the Presence of Retinal Extract." Current Eye Research 8 (9 1989): 871-881.

Miyazano, K., T. Okabe, A. Urabe, F. Takaku, and C.H. Heldin. "Purification and Properties of an Endothelial Cell Growth Factor from human Platelets." Journal of Biological Chemistry 262 (1987): 4098-4103.

Montesano, R., J.D. Vassali, A. Baird, R. Guillemin, and L. Orci. "Basic Fibroblast Growth Factor Induces Angiogenesis In Vitro." Proceedings of the National Academy of Science USA 83 (1986): 7297-7301.

Moscatelli, D., M. Presta, and D.B. Rifkin. "Purification of a Factor from Human Placenta that Stimulates Capillary Endothelial Cell Production, DNA Synthesis, and Migration." Proceedings of the National Academy of Science USA 83 (1986): 2091-2095.

Moses, M. A., J. Sudhalter, and R Langer. "Identification of an Inhibitor of Neovascularization from Cartilage." Science 248 (1990): 1408-1410.

Moses, M.A. and R. Langer. "A Metalloproteinase Inhibitor as an Inhibitor of Neovascularization." Journal of Cell Biochemistry 47 (1991): 1-6.

Muller, G., J. Behrens, U. Nussbaumer, P. Bohlen, and W. Birchmeier. "Inhibitory Activity of Transforming Growth Factor Beta on Endothelial Cells." Proceedings of the National Academy of Science USA 84 (1987): 5600-5604.

Mun, E.C., S.R. Doctrow, R. Carter, D. Ingber, and J. Folkman. "An Angiogenesis Inhibitor from the Cornea." Investigations in Ophthalmology and Visual Sciences 30 (1989): 151.

Murphy, R.C., J.R. Falck, S. Lumin, P. Yadagiri, J.A. Zirroli, M. Balazy, J.L. Masferrer, N.G. Abraham, and M.L. Schwartzman. "12(R)-Hydroxyeicosatrienoic Acid: A Vasodilator Cytochrome P-450 Dependent Arachidonate Metabolite from the Bovine Cornea Epithelium." Journal of Biological Chemistry 263 (1988): 17197-17202.

Needleman, P. and et al. "Arachidonic Acid Metabolism." Annual Review of Biochemistry 55 (1986): 69-102.

Nishizuka, Y. Science 233 (1986): 305.

Okamoto, T., S. Oikawa, and T. Toyota. "Absence of Angiogenesis-Inhibitory Activity in Aqueous Humor of Diabetic Rabbits." Diabetes 39 (1990): 12-16.

Patz, A. "Clinical and Experimental Studies on Retinal Neovascularization." American Journal of Ophthalmology 94 (6 1982): 715-743.

Rosenthal, R.A., J.F. Megyesi, W.J. Henzel, N. Ferrara, and J. Folkman. "Conditioned Medium from Mouse Sarcoma 180 Cells Contains Vascular Endothelial Growth Factor." Growth Factors 4 (1990): 53-59.

Ryan, T. "Factors Influencing the Growth of Vascular Endothelium in the Skin." British Journal of Dermatology 82 (1970): 99-111.

Samuelsson, B., S.E. Dahlen, J.A. Lindgren, C.A. Rouzer, C.N. Serhan. "Leukotrienes and Lipoxins: Structures, Biosynthesis and Biological Effects." Science 237 (1987): 1171-1176.

Schulze, R.R. "Rubeosis Iridis." American Journal of Opthalmology 63 (1967): 487-495.

Schwartzman, M.L., M. Balazy, J.L. Masferrer, N.G. Abraham, J.C. McGiff, and R.C. Murphy. "12(R)-HETE, a Cytochrome P450-Dependent Arachidonate Metabolite that Inhibits Na+-K+ ATPase in the Cornea." Proceedings of the National Academy of Science USA 84 (1987): 8125-8129.

Schweigerer, L., B. Malerstein, and D. Gospodarowicz. "Tumor Necrosis Factor Inhibits the Proliferation of Cultured Capillary Endothelial Cells." Biochemical and Biophysical Research Communications 143 (1987): 992-1004.

Shing, Y., J. Folkman, R. Sullivan, Butterfield C., J. Murray, and M. Klagsbrun. "Heparin Affinity: Purification of a Tumor-Derived Capillary Cell Growth Factor." Science 223 (1984): 1296-1299.

Sivalingham, A., J. Kenney, G.C. Brown, W.E. Benson, and L. Donoso. "Basic Fibroblast Growth Factor Levels in the Vitreous of Patients with Proliferative Diabetic Retinopathy." Archives of Opthalmology 108 (1990): 869-872.

Sullivan, R. and M. Klagsbrun. "Purification of Cartilage-Derived Growth Factor by Heparin Affinity Chromatography." Journal of Biological Chemistry 260 (1985): 2399-2401.

Sunderkotter, C., W. Bell, J. Roth, and C. Sorg. "Cellular Events Associated with Inflammatory Angiogenesis in the Mouse Cornea." American Journal of Pathology 138 (1991): 931-939.

Taraboletti, G., D. Roberts, L.A. Liotta, and R. Giavazza. "Platelet Thrombospondin Modulates Endothelial Cell Adhesion, Motility, and Growth: A Potential Angiogenesis Regulatory Factor." Journal of Cell Biology 111 (1990): 765-772.

Taylor, S. and J. Folkman. "Protamine is an Inhibitor of Angiogenesis." Nature 297 (1982): 307-312.

Thomas, K.A, M.C. Riley, S.K. Lemmon, N.C. Baglan, and Bradshaw R.A. "Brain Fibroblast Growth Factor." Journal of Biological Chemistry 255 (1980): 5517-5520.

Thomas, K.A., M. Rios-Candelore, and S. Fitzpatrick. "Purification and Characterization of Acidic Fibroblast Growth Factor from Bovine Brain." Proceedings of the National Academy of Science USA 81 (1984): 357-361.

Thomas, K.A. "Fibroblast Growth Factors." FASEB J 1 (1987): 434-440.

Verby, N.L. and N.J. Van Haeringen. "The Influence of a Fish Oil Dietary Supplement on Immunogenic Keratitis." Investigations in Opthalmology and Visual Science 31 (8 1990): 1526-1532.

Wagner, R.C. Advances in Microcirculation 9 (1980): 45-75.

Weidner, N., J. Semple, W. Welch, and J. Folkman. "Tumor Angiogenesis Correlates with Metastases in Invasive Breast Carcinoma." New England Journal of Medicine 324 (1991): 1-8.

West, D.C., I.N. Hampson, F. Arnold, and S. Kumar. "Angiogenesis Induced by Degradation Products of Hyaluronic Acid." Science 228 (1985): 1324-1326.

Williams, G.A., R. Eisenstein, B. Schumacher, K. Hsiao, and D. Grant. "Inhibitor of Vascular Endothelial Cell Growth in the Lens." American Journal of Opthalmology 97 (1984): 366-371.

Ziche, M., J. Jones, and P. Gullino. "Role of Prostaglandin E1 and Copper in Angiogenesis." Journal of the National Cancer Institute 69 (1982): 475-482.

HARVEY CUSHING / JOHN HAY WHITNEY
MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS DATE